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Dose-Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat

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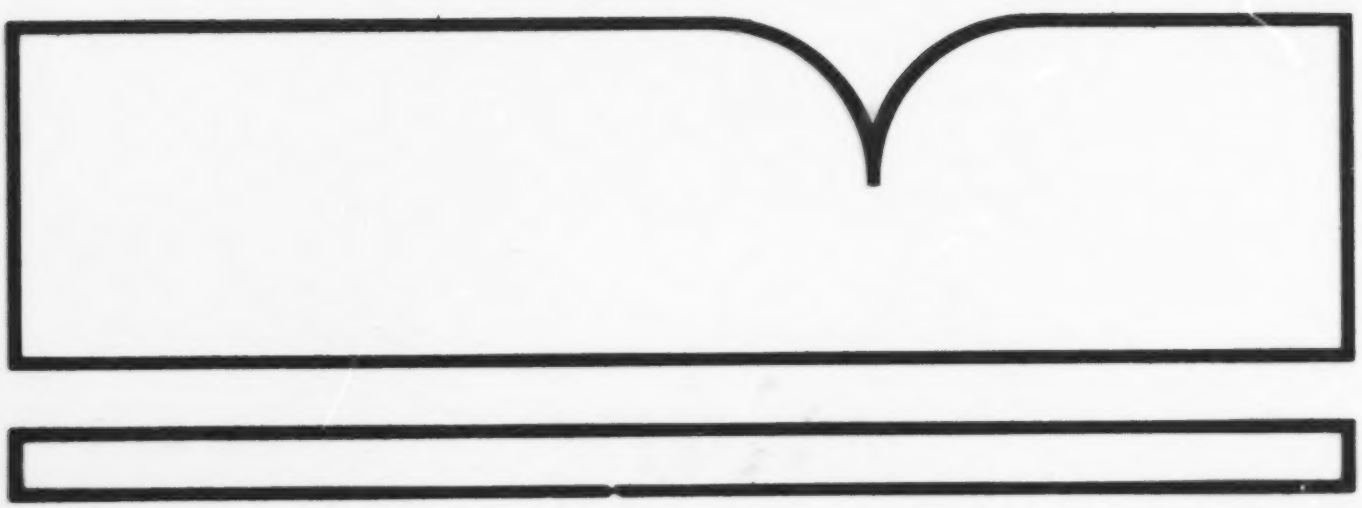

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16. ABSTRACT

In the present study rats were dosed from weaning, through puberty and gestation, to Day 15 of lactation with methoxychlor at 25, 50, 100, or 200 mg/kg/day. Morphological landmarks of puberty were measured, including the ages at vaginal opening, first estrus, and first estrous cycle in females and at preputial separation in males. In the female, estrous cyclicity, fertility, litter size, number of implantation sites, organ weights, and ovarian and uterine histology were also measured. The viability of the offspring (F1) and their fertility were evaluated using a continuous breeding protocol. Males were necropsied after breeding, the reproductive organs were weighed, and the cauda epididymal sperm counts were determined. One testis was used for histopathology, while the other was used to quantify interstitial fluid (IF) content, IF testosterone concentration, and testicular sperm production. Testosterone and androgen-binding protein were measured in the caput epididymis, and sperm motility and morphology were evaluated from a caudal sample. The serum and pituitary were saved for hormonal determinations. Methoxychlor accelerated the age at vaginal opening and first estrus, and the vaginal smears were cornified. Growth was retarded at 100 and 200 mg/kg/day and fertility was reduced when the females were bred with untreated or similarly treated males. In the highest-dose group, the mated females went from constant estrus into pseudopregnancy following mating, but they had no implants. In males, methoxychlor treatment markedly reduced growth, seminal vesicle weight, cauda epididymal weight, caudal sperm content, and pituitary weight. Puberty was delayed in the two highest-dosage groups. Testicular sperm measures were much less affected than caudal measures. Testis weight and histology were slightly affected, and testicular sperm production, sperm morphology, and motility were unaffected. Endocrine function of the testes and pituitary was altered by methoxychlor administration. Leydig cell testosterone production, in response to human chorionic gonadotropin challenge, was reduced and pituitary levels of prolactin, thyroid-stimulating hormone (TSH), and follicle-stimulating hormone (FSH) were altered. In contrast, serum levels of prolactin, FSH, and luteinizing hormone were unaffected. Serum TSH was reduced by 50% of control at 100 and 200 mg/kg/day, while pituitary levels were increased. Gonadotropin-releasing hormone concentration in the mediobasal hypothalamus was also elevated. In spite of the many reproductive alterations, the fertility of treated males was not reduced when they were mated with untreated females. Growth and viability of the offspring (F1) from the 50 mg/kg/day treatment group were normal, but in the females, vaginal opening was accelerated, estrous cyclicity was abnormal in the rats during middle age, and fecundity was reduced. © 1989 Society of Toxicology.

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A Dose–Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat

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A Dose–Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat. GRAY, L. E., JR., OSTBY, J., FERRELL, J., REHNBERG, G., LINDER, R., COOPER, R., GOLDMAN, J., SLOTT, V., AND LASKEY, J. (1989). *Fundam. Appl. Toxicol.* **12,** 92–108. In the present study rats were dosed from weaning, through puberty and gestation, to Day 15 of lactation with methoxychlor at 25, 50, 100, or 200 mg/kg/day. Morphological landmarks of puberty were measured, including the ages at vaginal opening, first estrus, and first estrous cycle in females and at preputial separation in males. In the female, estrous cyclicity, fertility, litter size, number of implantation sites, organ weights, and ovarian and uterine histology were also measured. The viability of the offspring (F1) and their fertility were evaluated using a continuous breeding protocol. Males were necropsied after breeding, the reproductive organs were weighed, and the cauda epididymal sperm counts were determined. One testis was used for histopathology, while the other was used to quantify interstitial fluid (IF) content, IF testosterone concentration, and testicular sperm production. Testosterone and androgen-binding protein were measured in the caput epididymis, and sperm motility and morphology were evaluated from a caudal sample. The serum and pituitary were saved for hormonal determinations. Methoxychlor accelerated the age at vaginal opening and first estrus, and the vaginal smears were cornified. Growth was retarded at 100 and 200 mg/kg/day and fertility was reduced when the females were bred with untreated or similarly treated males. In the highest-dose group, the mated females went from constant estrus into pseudopregnancy following mating, but they had no implants. In males, methoxychlor treatment markedly reduced growth, seminal vesicle weight, cauda epididymal weight, caudal sperm content, and pituitary weight. Puberty was delayed in the two highest-dosage groups. Testicular sperm measures were much less affected than caudal measures. Testis weight and histology were slightly affected, and testicular sperm production, sperm morphology, and motility were unaffected. Endocrine function of the testes and pituitary was altered by methoxychlor administration. Leydig cell testosterone production, in response to human chorionic gonadotropin challenge, was reduced and pituitary levels of prolactin, thyroid-stimulating hormone (TSH), and follicle-stimulating hormone (FSH) were altered. In contrast, serum levels of prolactin, FSH, and luteinizing hormone were unaffected. Serum TSH was reduced by 50% of control at 100 and 200 mg/kg/day, while pituitary levels were increased. Gonadotropin-releasing hormone concentration in the mediobasal hypothalamus was also elevated. In spite of the many reproductive alterations, the fertility of treated males was not reduced when they were mated with untreated females. Growth and viability of the offspring (F1) from the 50 mg/kg/day treatment group were normal, but in the females, vaginal opening was accelerated, estrous cyclicity was abnormal in the rats during middle age, and fecundity was reduced. © 1989 Society of Toxicology.

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Presently, no-observed-effect levels (NOELs) for reproductive toxicity are set using data on fertility, litter size, and litter viability—endpoints that are often insensitive indices of reproductive health. For example, a number of studies, using both male rats and male mice, have found that fertility is normal even when sperm counts are reduced by 90% (Meistrich, 1982). In another study, male mice with partial testicular agenesis, induced by *in utero* exposure to a dye, reproduced continuously at normal levels over an 11-month period (Gray *et al.*, 1984). In the female, so little data exist for endpoints other than fertility that it is impossible to determine if adverse effects occur at doses below those that alter fertility. Thus, alternate protocols that emphasize endpoints in addition to fertility need to be developed. While some consideration has been given to including more sophisticated assessments of testicular histology (Lamb and Chapin, 1985; Russell, 1983) and sperm function (Linder *et al.*, 1986; Overstreet, 1984), effects on other reproductive organs in the male (like pituitary and endocrine function) and additional reproductive measures in females (such as estrous cyclicity) are rarely reported.

We are using a protocol that incorporates indices of puberty in the rat along with other morphological and physiological indices of reproductive status (Gray *et al.*, 1985). Secondary sex characteristics develop in a predictable sequence during puberty and alterations of this process are easily identified. The present study was designed to develop dose–response data for a number of different endpoints following subchronic exposure to the known reproductive toxicant methoxychlor during the pubertal, young adult, gestational, and lactational stages of life. Methoxychlor is a weakly estrogenic pesticide that has been shown to alter fertility in male and female rats (Bulger *et al.*, 1978). In the male, it alters spermatogenesis at high doses (Tullner and Edgcomb, 1962). In the female, it produces a state of constant estrus, blocks implantation (Bal and Mungkornkarn, 1978), and alters sex differentiation (Harris *et al.*, 1974) such that perinatally exposed females are infertile as adults. However the relative sensitivities of the two sexes and that of the fetal rat to methoxychlor-induced alterations of fertility are unknown. Furthermore, the dose–response characteristics of other reproductive endpoints at doses of methoxychlor below those that result in infertility are also unknown. Identification of the susceptible population, the target organ, and the dose–effect relationships for more sensitive reproductive endpoints, as compared to the examination of fertility alone, would allow for a more scientifically based extrapolation of the effects seen in studies using rats to potential effects in humans. The present study describes the reproductive alterations induced by subchronic administration of methoxychlor (at 25, 50, 100, and 200 mg/kg/day). Treatments were initiated at weaning and continued through puberty, young adulthood, breeding, gestation, and lactation.

METHODS

Animals

In the present study 90-day-old pregnant Long–Evans hooded rats were received from Charles River Breeding Laboratory on Day 3 of gestation. Upon receipt, dams were housed individually in clear plastic cages (20 × 25 × 47 cm) with laboratory-grade pine shavings as bedding. Animals were maintained on Purina Rat Chow (5001) and tapwater *ad libitum*, in a room with a 12:12 photoperiod, temperature of 20–24°C, and relative humidity of 40–50%. Methoxychlor (Lot m-1501, 90% purity) was acquired from Sigma Chemical Company. Laboratory-grade corn oil was the vehicle chosen to prepare all dosing solutions.

On Postpartum Day 6 the dams were weighed and litters were culled to four males and four females per litter and weighed by sex. On Postpartum Day 21, pups were weighed, weight ranked by sex, and assigned to treatment groups in a manner that provided each treatment with equal means and variances in body weight.

Dosing

After being assigned to treatment groups at weaning, rats were dosed by gavage with 5 μl of corn oil/g body wt until Day 45, at which time the volume was decreased to 2.5 μl of oil/g body wt. This dosing regimen included

twice-weekly dose adjustments based on weight changes and continued throughout the study. In females, dosing was discontinued in Block 1 after parturition, but it was continued through Postpartum Day 15 in Blocks 2 and 3. Males were dosed from weaning to necropsy.

Experimental Design

The study was conducted in three blocks (Fig. 1). In Block 1, there were three treatment groups: 40 control animals—20 females paired with 20 males; 30 methoxychlor (100 mg/kg/day)-treated animals—15 females paired with 15 males; and 30 methoxychlor (200 mg/kg/day-treated) animals—15 females paired with 15 males. Rats were housed in bisexual pairs from weaning until the males were necropsied at 77–80 days of age.

Block 2 consisted of four treatment groups: untreated controls; treated controls; and two methoxychlor-treated groups (25 and 50 mg/kg/day). Eight females were paired at weaning with eight similarly dosed males in each of the treatment groups until the males were necropsied at 80–85 days of age.

In the third block, methoxychlor-treated rats were bred with untreated rats (from the same cohort) to determine which sex was infertile. In this block there were 12 controls/sex, while 8 rats/sex received methoxychlor at 100 mg/kg/day and another 8/sex were dosed with 200 mg/kg/day. The rats in this block were housed in unisexual pairs from Day 20 until Day 78, when they were placed in wire-bottom cages with an untreated rat of the opposite sex. At this time all 104 females were checked daily for sperm and plugs in the vagina. Vaginal smears were also taken to confirm pregnancy and copulatory plugs were counted. On Day 94 breeding was terminated, males were returned to their cages, and the females were housed individually. On Days 97 to 100 the males were killed by decapitation and necropsied.

Pubertal Indices in the Female

Morphological changes associated with puberty were measured in female rats starting on Postpartum Day 21. The criteria used to mark the onset of puberty were the ages at the opening of the vagina, the first estrous smear, and the onset of regular estrous cycles. The onset of hormonal cyclicity at sexual maturity has been shown to be highly correlated with these parameters in the female rat (Parker and Mahesh, 1976). Following vaginal opening, vaginal estrous cycles were monitored until parturition. The percentage of vaginal smears with leukocytes from vaginal opening until pregnancy was calculated to quantify methoxychlor-induced periods of persistent cornification.

Breeding

The females were judged to be pseudopregnant if they had vaginal smears with leukocytes for at least 10 days in a row. The presence of sperm in the vaginal smear or a copulatory plug in the vagina was noted (indicating that breeding had taken place), and blood in the smears was also noted as this is often associated with reproductive failure due to fetal resorption during pregnancy or the degeneration of a deciduoma at the end of pseudopregnancy. In the first two blocks the female rats were checked daily for delivery of litters starting on Day 64. The dams' weight at parturition and on Postpartum Day 5 was measured; Postpartum Day 1 weight was also measured in Blocks 2 and 3.

Female Necropsy

After being weighed, Block 1 dams were killed using CO_2 and necropsied on Postpartum Day 5. Block 2 and 3 dams were kept with their litters and necropsied on Day 25. The data collected at necropsy included body weight, pituitary weight, and number of implantation sites or, if pregnant, number of fetuses. Ovarian weight was measured in Block 2 and 3 dams, and the uterus and ovaries were fixed in Bouin's solution, washed and stored in 70% alcohol, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin. In the present study all tissues were examined by Experimental Pathology Laboratories, Inc. (Research Triangle Park, NC) for treatment-induced histopathological lesions.

Offspring (F1)

The litters were weighed, and the live and dead pups were counted on Postnatal Days 1 and 5. In Block 1, the pups were euthanized on Postnatal Day 5. In Block 2, the pups were weaned on Postnatal Day 25 when the offspring were weighed. Then two males and two females were randomly chosen from each control and methoxychlor litter. All other pups were euthanized. These littermates were housed one breeding pair per cage, and their breeding success was monitored continuously until 11 months of age, at which time the males were necropsied. The females' estrous cyclicity was monitored for 1 month and then they were necropsied.

Male Pubertal Indices

Preputial separation, an androgen-dependent morphological change associated with puberty in the male rat, is an easily identifiable index of male pubertal development (Korenbrot *et al.*, 1977). Measurement of this index began on Postpartum Day 35. Separation is considered complete when the prepuce can be completely retracted to expose the glans penis.

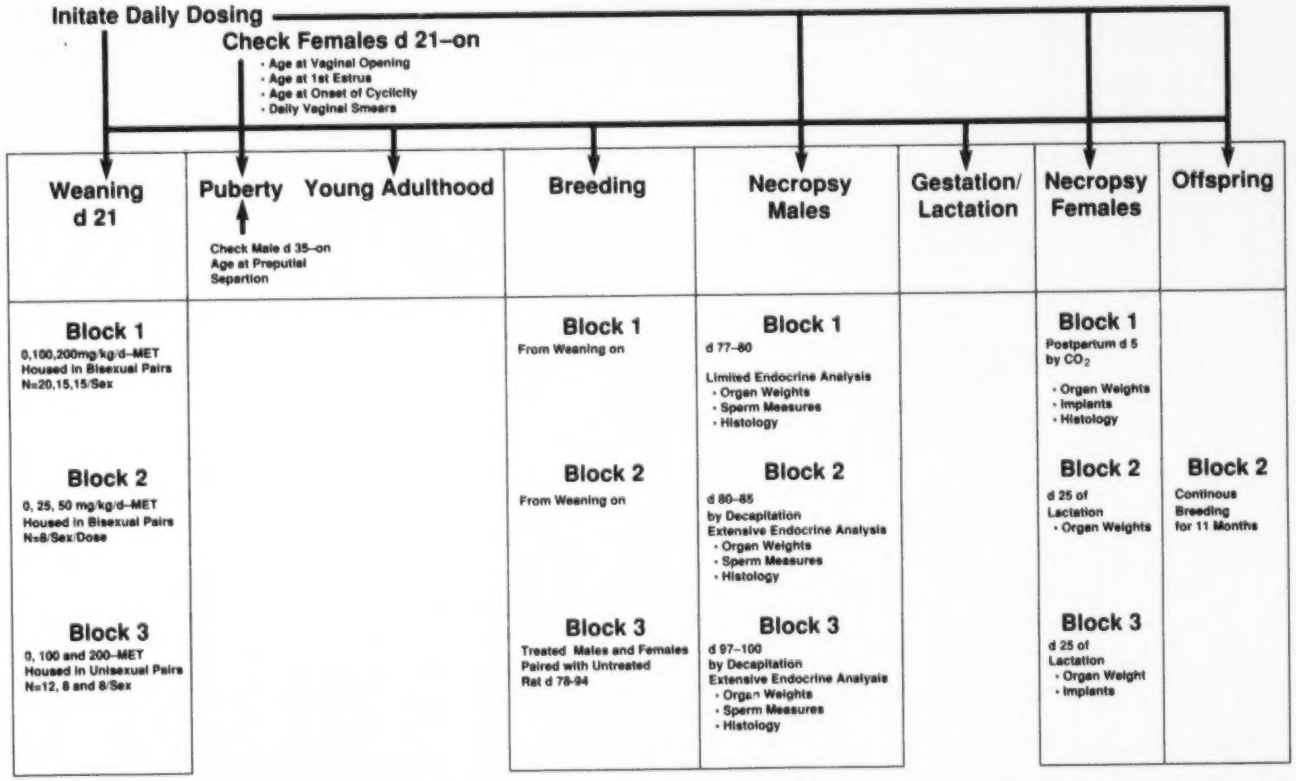

FIG. 1. A summary of the experimental design of the present study which assessed the effects of daily oral dosing of methoxychlor at 0, 25, 50, 100 or 200 mg/kg/day on the reproductive development of male and female Long–Evans hooded rats. Male rats were dosed from weaning through puberty, young adulthood, and breeding and then necropsied as indicated. Females were also dosed through gestation and lactation. The offspring were not dosed directly but were potentially exposed to the compound *in utero* and in the milk. The study was conducted in three blocks and the doses, sample sizes, and methods for each block are presented in this figure.

Male Necropsy

All Block 1 males were necropsied on Days 77–80. They were anesthetized with Nembutal (50 mg/kg) and blood was drawn by cardiac puncture. In Blocks 2 (Days 80–85) and 3 (Days 97–100) the males were brought to an animal room near our laboratory the day before their necropsy for a 24-hr acclimation period. They were killed by decapitation within 15 sec of removal from their cages in another room to avoid stress effects on the hormones, especially prolactin. Blood was collected in a 9-ml serum separation tube and left at room temperature for 30 min. Serum was collected by centrifuging the blood for 10 min at 3000 rpm and 5°C, then aliquoted into five 1.5-ml autoanalysis cups, and stored at −40°C. Serum was subsequently analyzed for hormonal content which included testosterone, androgen-binding protein, follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, and thyroid-stimulating hormone (TSH).

Weights measured at necropsy included body, testes, liver, kidney, adrenal (an estrogen-stimulated gland), seminal vesicle (an androgen-sensitive tissue), head and tail of the epididymis, and pituitary. One testis was fixed in Bouin's solution after a cross section of fresh tissue had been removed for assay of glutathione (GSH) levels, stored in 70% alcohol, and saved for microscopic examination (conducted by Experimental Pathology Laboratories, Inc. In addition, the testes of Block 1 males, including the control group and the groups administered 100 and 200 mg/kg/day methoxychlor, were reexamined by Pathco Inc. (Research Triangle Park, NC). The portion of the testis used for GSH levels was homogenized, frozen at −60°C, and assayed later using a Beckman DU-7 spectrophotometer following an enzymatic cycling method as described by Slott and Hales (1987). Testicular GSH depletion is correlated with a decrease in spermatogenesis.

One cauda epididymis was weighed and a small sample of caudal sperm was placed on a slide in Dulbecco's phosphate-buffered saline that had been warmed to 37°C. The percentage of motile sperm was estimated, and cellular debris, clumps of immotile sperm, and abnormal sperm were noted if present in the sample. The cauda epididymis was then placed in a 20-ml glass scintillation vial and minced with 2 ml of 10% phosphate-buffered formalin. Eight milliliters more of formalin was added and the sample was vortexed for 1 min. The sample was vortexed daily for five consecutive days. This 1:10 dilution was further diluted to produce a sperm count of between 10,000 and 20,000/0.5 ml of sample. The sperm were counted on a ZBI Coulter Counter with a c-1000 channelyzer (Carter *et al.*, 1984). The settings were as follows: Coulter counter-sample volume 0.5 ml, aperture size 70 μm, aperture current 1; amplification 0.55, gain 6.5, upper threshold 55, lower threshold 30; C-1000 channelyzer-base channel threshold 5, window width 100. At least three separate counts of each sample were taken and the mean value was used. A hemocytometer count was made on at least 10% of the samples to check the accuracy of the Coulter counter and to look for cellular debris. In a study with low counts or with considerable debris present in the samples, all samples should be counted using only the hemocytometer.

The other testis and pituitary were utilized for hormonal content. In the testis, testosterone and androgen-binding protein were measured. In the pituitary, prolactin, FSH, LH and TSH concentrations were determined. One caput epididymis was used for an androgen-binding protein and testosterone determination.

Testicular interstitial fluid (IF) was collected using a modification of procedures described by Sharpe (1979) and Turner *et al.* (1984). One testis and caput epididymis was removed, cleaned of extraneous material, and weighed. A small incision was made in the caudal end of the testicular capsule and 2-O silk was attached to the other end of the testis with an auto clip. The testis was suspended slightly above the hub of a 10-ml syringe by taping the silk to the outside of the syringe. A preweighed 1.5-ml microfuge tube was attached to the hub of the syringe. The testis was then centrifuged at 30*g* for 45 min at 4°C, the microfuge tube containing the collected IF was weighed, and 200 μl of phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA) was added. After centrifugation to remove cellular debris, the supernatant was stored at −40°C.

The same testis was used for human chorionic gonadotropin (hCG)-stimulated testosterone synthesis *in vitro* and then for sonication-resistant spermatid head count as described by Cassidy *et al.* (1983). For the testosterone assay the decapsulated testis was placed in 2.5 ml of medium 199/0.3% BSA (pH 7.4) containing 10m*M* Hepes in a 20-ml scintillation vial. This was incubated in a water bath at 37°C for 1 hr, at which time a 100-μl sample was taken (0 hr) and 100 IU of hCG in 0.1 ml was added. At 1, 2, and 3 hr after hCG stimulation, additional 100-μl samples were taken. The caput epididymides were frozen for subsequent ABP assay. The thawed caput was homogenized in cold TEG buffer (20 mM Tris, 2.0 mM EDTA, and 10% glycerol, pH 7.4) using a tissue-to-buffer ratio (w/v) of 1:9. The homogenates were centrifuged at 30,000*g* for 1 hr and the supernatant was diluted and assayed for ABP. Reagents for the ABP were provided by the National Hormone and Pituitary Program. The general assay procedures of Gunsalus *et al.* (1978) were utilized. Pure rat ABP was iodinated using the iodogen method of Fracker and Speck (1978). The ABP values are expressed in terms of the NICHD WR-R1. The sensitivity of the assay was 0.5 ng/tube.

Testosterone was measured using a No-Extraction Coat-A-Count RIA kit obtained from Diagnostic Products Company (Los Angeles, CA). The intra- and interassay variability was 8 and 10%, respectively. LH, FSH, and prolactin (PRL) kits were kindly supplied by the National Hormone and Pituitary Program. Serum LH, FSH, and PRL RIAs were performed using a modification of the procedure of Niswender *et al.* (1969). Carrier-free ^{125}I was obtained from New England Nuclear (Bos-

ton, MA). Pure rat LH (NIADDK-rLH-I-6), FSH (NIADDK-rFSH-I-3), and PRL (NIAMDD-rPRL-I-5) were iodinated using the chloramine-T method (Greenwood *et al.*, 1963). The LH values are expressed in terms of NIADDK-rLH-RP2. The sensitivity of the LH RIA was 15 pg/tube. The antiserum to LH was NIADDK-anti-rat-LH-S-9 at a dilution of 1:40,000. FSH values are expressed in terms of NIADDK-rFSH-RP2. The sensitivity of FSH RIA was 3 pg/tube. The antiserum to FSH was NIADDK-anti-rat-FSH-S-11 at a dilution of 1:2500. PRL values are expressed in terms of NIAMDD-rPRL-RP-3. The sensitivity of the assay was 32 pg/tube. The antiserum to PRL was NIAMDD-anti-rat-PRL-S-9 at a dilution of 1:12,500.

The second cauda epididymis was used for a quantitative assessment of progressive sperm motility and sperm morphology. The relative number of progressively motile cauda sperm were measured using the methodology described in detail by Linder *et al.* (1986). This method of determining motility is considerably more precise than that qualitative estimate described earlier, but unlike the other procedure, very low sperm counts or clumps of sperm and debris in the sample preclude quantitative analysis. Caudal sperm from the motility assay were fixed in approximately 5% formalin for assessment of sperm morphology. Wet preparations were examined by phase-contrast microscopy. Two hundred sperm heads, or intact sperm, per animal were categorized as normal intact cells with no abnormalities or as abnormal. The abnormalities include (1) isolated heads or intact (head plus tail) cells with atypical head shape; (2) isolated heads of normal shape; or (3) intact (head plus tail) cells with bent, coiled, or degenerating flagellae.

Statistical Analysis

The parametric data were analyzed by block using a regression analysis, the General Linear Models procedure (PROC GLM), available in the 1982 edition of the Statistical Analysis System (*SAS User's Guide*, 1982). All endocrine data were log transformed to normalize the distributions and eliminate heterogeneity of variance between the groups. When significant ($p < 0.05$) treatment effects were detected in the ANOVA, then two-tailed *t*-test comparisons were made using the LSMEANS option of the PROC GLM. Percentage data (e.g., fertility) were analyzed using the χ^2 option of the Proc Freq, also available on SAS, and individual comparisons were made using Fisher's exact test.

RESULTS

Growth

Growth rates were depressed by methoxychlor treatment, with the male being more affected than the female (Table 1). In the female, growth was not retarded at 25 and 50 mg/kg/day (Table 1), and the significant effects at 100 mg/kg/day (down 12% at necropsy, Table 2A) and 200 mg/kg/d (down 20%) were less than those seen in males at equivalent doses. In the male this effect was significant at doses of 25 (down 11% at necropsy, Table 3), 50 (down 17%), 100 (down 23%), and 200 (down 32%) mg/kg/day.

Female Pubertal Indices

Methoxychlor treatment accelerated the ages at vaginal opening and first estrous smear at all doses (Table 2A). The onset of cyclicity was accelerated at 25 mg/kg/day, normal at 50 and 100 mg/kg/day, while puberty was delayed at 200 mg/kg/day because the females never cycled normally (Table 2A).

Estrous Cyclicity

The percentage of vaginal smears that contained leukocytes from the time of vaginal opening until pregnancy was reduced at 50, 100, and 200 mg/kg/day methoxychlor, indicating that this weak estrogen was increasing the frequency of cornified smears (Table 2A). Many of the females administered 200 mg/kg/day never cycled and remained in a state of constant vaginal cornification, while others had prolonged cycles with extended periods of estrus.

Breeding and Fertility/Female

Sperm was detected in the vaginal smears of the majority of the females from all dose groups, even though many of the females in the highest dose groups were acyclic. In the first block, 12 of the 15 females administered methoxychlor at 100 mg/kg/day bred with treated males, as indicated by sperm-positive vaginal smears, but only 3 of these became pregnant (Table 2A). Not only were the other

TABLE 1

EFFECTS OF DAILY ADMINISTRATION OF METHOXYCHLOR ON BODY WEIGHTS OF MALE AND FEMALE RATS[a]

Age (Weeks)	Block 2				Block 1		
	Oil	None	M25	M50	Oil	M100	M200
Males							
3	43	43	45	45	51	51	51
4	71	76	77	77	87	86	84*
5	119	124	126	123	134	127*	118**
6	169	181	172	166	178	158**	150**
7	228	238	225	217	206	179**	166**
8	265	293	269	257	287	232**	211**
9	317	333	302	285**	327	257**	233**
10	355	367	329*	309**	361	277**	245**
11	389	399	350**	328**	383	294**	260**
Females							
3	42	43	43	43	49	49	49
4	70	71	70	71	82	79**	77**
5	108	109	108	107	121	113**	107**
6	140	140	133	135	146	136**	130**
7	161	167	163	157	175	161**	152**
8	187	195	185	185	202	184**	171**
9	196	215	199	199	225	200**	185**
10	220	239	229	221	238	208**	189**

[a] Values are mean weights (8 to 15 animals per group) in grams.
* $p < 0.05$.
** $p < 0.01$ less than sham control.

9 infertile, they also remained in constant estrus after breeding. In the females treated with methoxychlor at 200 mg/kg/day, 5 were found to have sperm in their vaginal smears, whereas 9 did not (2 died). Four of these five failed to become pregnant or pseudopregnant.

In the second block many of the females had litters and were pregnant for the second time when they were necropsied. For this reason, the ovarian weight and implant data, which were recorded for only the nonpregnant females, represent a subsample of the females and are not presented.

In the third block, treated females bred with untreated males, most females had sperm-positive vaginal smears, indicating that mating had occurred. In addition, all of the females were either pregnant (5/8 at 100 and 0/8 at 200 mg/kg/day) or pseudopregnant.

Infertility resulted either from failure of fertilization of the ova or from inability of the egg to implant, as those females that were not pregnant had no implantation sites. Methoxychlor treatment at 50 ($p < 0.1$) and 100 ($p < 0.01$) mg/kg/day also decreased the number of pups born per breeding female (Table 2A).

Female Necropsy Data

Methoxychlor treatment at 100 and 200 mg/kg/day caused atrophy of the entire reproductive tract; five of eight females in the high-dosage group (Block 3) had no visible corpora lutea on the surface of the ovary. In the second block ovarian weights were not affected by methoxychlor administration at 25 or 50 mg/kg/day (Table 2B).

Female/Histology

In Blocks 1 and 3, treatment-related changes were observed in female rats that re-

TABLE 2A

EFFECTS OF DAILY ADMINISTRATION OF METHOXYCHLOR ON PUBERTAL DEVELOPMENT, FERTILITY, AND REPRODUCTIVE ORGAN WEIGHTS IN THE FEMALE RAT

	Block 2				Block 1			Block 3		
	Oil	None	M25	M50	Oil	M100	M200	Oil	M100	M200
Age at V0 (days)	32	33	26*	25*	34	26*	25*	32	25*	24*
Age at first estrus	33	33	26*	25*	34	27*	25*	33	25*	24*
Age at first cycle	34	37	29*	36	35	39	63*	36	42	89*
Weight at V0 (g)	97	100	64*	61*	116	70*	65*	113	71*	66*
% of smears with leukocytes	62	66	63	39**	68	42*	13*	65	34*	5*
No. of cornified smears prior to first cycle	1.8	1.3	3.7	10.8*	1.2	13.6*	42*	0.8	15*	45.7*
Fertility (%)	100	100	100	100	100	20*	0*	83	50*	0*
Age of parturition	82.6	85.9	80.0	83.9	81	87		100	105	
Postpartum weight (g)	235	259	231	238	ND[a]	ND	ND	281	248	
No. live pups Day 1	9.3	11.1	9.7	7.2	11.1	5.0**	0*	15.0	5.7*	0*
Pup weight (g) Day 1	5.84	5.96	5.77	7.07**	5.9	6.7		5.6	7.0**	
No. live pups Day 5	7.9	10.0	9.1	5.5	10.2	5.0	0*	12.3	4.7*	0*
Pup weight (g) Day 5	11.2	10.5	9.9	12.9	10.5	11.6		8.8	12.0	
Pup weight (g) Day 25	71	69	67	72	ND	ND	ND	ND	ND	ND
Dam weight (g) Day 25	275	283	272	259	ND	ND	ND	ND	ND	ND

[a] No data.
* $p < 0.01$.
** $p < 0.05$.

TABLE 2B

EFFECTS OF DAILY ADMINISTRATION OF METHOXYCHLOR ON ORGAN WEIGHTS AT NECROPSY IN THE FEMALE RAT

	Block 2				Block 1			Block 3		
Block	Oil	None	M25	M50	Oil	M100	M200	Oil	M100	M200
Body weight (g)	268	282	267	260	276	237*	212*	297	287	243**
Liver weight (g)	ND[a]				ND			17	15	11*
Kidney weight (g)	ND				ND			1.1	1.0	0.96**
Adrenal weight (mg)								64	87*	83**
Pituitary weight (mg)	8	10	10	9	ND	ND	ND	12	10**	8*
Ovarian weight (mg)					ND	ND	ND	109	84**	58*
No. implants/ pregnant dam					13	7**	—	14	9	—
Histopathology of ovaries (% affected)	0	0	0	0	0	40	100	0	50	63
No. of rats	8	7	8	8	19	15	15	12	8	8

[a] No data.
* $p < 0.01$.
** $p < 0.05$.

ceived either 100 or 200 mg/kg/day methoxychlor (Table 2B). These changes consisted of decreased corpora lutea; the most severe reductions were observed for females receiving 200 mg/kg/day methoxychlor. The reduction in corpora lutea was associated with an increased number of granulosa–thecal cells in the interstitial tissue, an increased number of granulosa–thecal cells in the interstitial tissue, and an increased number of atretic ovarian follicles. In Block 2, no differences in histologic appearance were noted between ovaries and uteri of rats given 25 and 50 mg/kg/day methoxychlor.

Male Pubertal Indices

The age at prepubertal separation (PPS) was delayed (Table 3) by methoxychlor treatment at 100 and 200 mg/kg/day, but not at lower doses. In addition, the effect was more dramatic in the first block than in the third, although the delay was significant in both blocks. The weight at PPS was increased in Block 1 males dosed at 200 mg/kg/day.

Fertility/Male

In the third block, when males were mated with untreated females, all breeding pairs were fertile and methoxychlor treatment did not reduce the numbers of implants (Table 3).

Male Necropsy Data

In general, the testes were much less affected than body weight, cauda epididymal weight, epididymal sperm content, and weight of the seminal vesicle, an androgen-dependent organ (Table 3). Testis weight was only slightly reduced (11%) at 200 mg/kg/day, whereas cauda epididymal weight, caudal sperm content, and seminal vesicle weight were affected at a lower dose (50 mg/kg/day) and were reduced by about 50% in the high-dose group (Table 3). Pituitary weight was reduced in the males treated with methoxychlor at 200 mg/kg/day. Adrenal weight, an estrogen-stimulated gland, was increased at 100 and 200 mg/kg/day. Progressive sperm motility, sperm morphology, and testicular sperm head count were not affected by methoxychlor treatment at any dose.

Methoxychlor treatment induced hyperprolactinemia in two of eight males at 200 mg/kg/day (an effect that was not statistically significant), whereas pituitary content of prolactin was doubled in all the treated groups (Table 4). In contrast, serum and pituitary levels of LH were unaffected at any dose, while pituitary, but not serum, FSH content was increased. Serum testosterone levels were reduced at 100 mg/kg/day and were undetectable at 200 mg/kg/day in Block 1 but not in Block 3. Although serum testosterone was not reduced in Block 3, there were significant alterations of Leydig cell function (Table 5); IF testosterone was reduced at 100 mg/kg/day, and hCG-stimulated testosterone production was reduced at 100 and 200 mg/kg/day (Table 5). In addition, epididymal testosterone concentration was reduced by 50% of control in both of the higher-dosage groups. Levels of androgen-binding protein, a Sertoli cell product, were not different from control levels in the interstitial fluid and caput epididymis, but serum levels were elevated at 200 mg/kg/day.

Histology/Male

The testes of rats in all dosage groups below 200 mg/kg/day were histologically normal. The administration of methoxychlor at 200 mg/kg/day did not cause any severe testicular lesions, but two rats in the high-dose group from the first block had clearly observable lesions. One animal had minimal seminiferous tubular atrophy and another had mild hypospermatogenesis which affected the majority of tubules. These lesions were not accompanied by any degenerative changes but simply appeared as a reduction in spermatozoa present per cross section of tubule.

TABLE 3

EFFECTS OF DAILY ADMINISTRATION OF METHOXYCHLOR AT 25, 50, 100, AND 200 mg/kg/day ON REPRODUCTIVE ENDPOINTS IN THE MALE RAT

	Block 2				Block 1			Block 3		
	Oil	None	M25	M50	Oil	M100	M200	Oil	M100	M200
Age at PPS (days)	42	41	41	43	40.4	43.8*	53*	39.5	44**	45.9**
Weight at PPS (g)	175	181	172	173	178	168	198**	195	194	199
Necropsy										
Body weight (g)	422	422	362*	342*	382	286**	255**	489	348**	335**
Liver weight (g)	16.9	16.4	16.3	15.2	15.5	12.7*	11.3*	24.7	16.0**	15.5**
Kidney weight (g)	1.50	1.57	1.46	1.34	1.35	1.10**	1.03**	1.75	1.31**	1.23**
Adrenal weight (mg)	50	56	47	48	40.2	52.4**	50.0**	51.4	74.4	78.8*
Seminal vesicle weight (g)	1.33	1.50	1.25	1.08*	1.19	0.72**	0.34**	1.73	0.89**	0.71**
Testis weight (g)	1.76	1.73	1.62	1.58	1.59	1.50	1.31**	1.87	1.66*	1.67*
Cauda epididymis weight (mg)	233	257	217	197*	202	165**	119**	300	224**	198**
Caudal sperm count	86×10^6	97	72	62**	79	63**	37**	138	91**	82**
Sperm motility index (%)	47	59	54	51	ND	ND	ND	53	60	47
Testicular sperm heads	237×10^6	202	220	199	ND	ND	ND	197	166	222
Abnormal sperm (%)	1.5	2.0	2.0	3.5	ND	ND	ND	6.5	1.0	2.5
Pituitary weight (mg)	9.9	10.8	9.8	9.2	9.8	8.7	7.9**	10.7	8.6**	8.3**
Age at necropsy (days)	83	83	83	83	77	77	77	96	96	96

[a] No data.
* $p < 0.05$.
** $p < 0.01$.

Offspring

In the control females, vaginal opening occurred at 32.5 days; they were all fertile, producing 6.7 litters and 78.3 pups from Day 35 to Day 253, and at 11 months of age 60% displayed normal estrous cycles (Table 6). In contrast, vaginal opening was accelerated by about 6 days, only 60% of the treated (50 mg/kg/day) offspring bred, resulting in 3.4 litters and 33 pups per female, and none of the females had normal cycles at 11 months of age. In the male offspring, body, liver, kidney, adrenal, pituitary, cauda epididymal, and seminal vesicle weights were not affected by methoxychlor treatment. Testis weight and epididymal sperm counts were less than control values (1.80 g for control vs 1.66 g for methoxychlor; 150 million sperm for control vs 119 for methoxychlor) but these differences were not significant ($0.05 < p < 0.10$).

DISCUSSION

In the present study we evaluated the effects of methoxychlor on pubertal development and reproductive function in the male and female rat. Methoxychlor mimicks estrogen *in vivo* and *in vitro*, and as a consequence of this activity (in the present study), methoxychlor accelerated vaginal opening, cycles were in constant estrus, ovarian luteal function was inhibited, and implantation was blocked. In the male, it inhibited somatic growth and accessory gland weight, elevated pituitary and serum prolactin levels, and suppressed testicular Leydig cell function. The

TABLE 4

EFFECTS OF DAILY ADMINISTRATION OF METHOXYCHLOR AT 25, 50, 100 OR 200 mg/kg/day ON SERUM AND PITUITARY LEVELS OF THE PITUITARY HORMONES, MEASURED IN BLOCKS 2 AND 3

	Block 2				Block 3		
	Oil	None	M25	M50	Oil	M100	M200
Serum concentration (ng/ml)							
Prolactin	3.6	6.5	6.0	8.5	3.7	2.0	13.2
LH	0.54	0.52	0.56	0.48	0.48	0.32	0.52
FSH	7.9	6.8	7.7	8.7	8.9	7.8	9.5
TSH					6.3	2.7*	2.98*
Pituitary concentration (ng/mg)							
Prolactin	1654	1869	3128*	3200*	1936	3281*	3032**
LH	615	487	503	620	681	725	793
FSH	305	336	240	252	592	715**	781*
TSH					1579	1987**	2022**

* $p < 0.01$.
** $p < 0.05$ different from concurrent control.

TABLE 5

EFFECTS OF SUBCHRONIC TREATMENT WITH METHOXYCHLOR AT 25, 50, 100, OR 200 mg/kg/day ON THE TESTICULAR ENDOCRINE PROFILE OF THE MALE RAT

	Block 2				Block 3		
	Oil	None	M25	M50	Oil	M100	M200
IF[a] weight (mg)	95	87	74	70	91	91	85
IF T (ng/ml)	187	277	252	346	298	70*	244
Serum T (ng/ml)	2.1	4.0	3.4	5.2	2.8	0.7*	2.3
Epididymal T (ng/epididymis)					3.9	1.2*	1.5*
Seminiferous tubular T (ng/ml)					761	546	721
hCG-stimulated T (ng/testis)							
0 hr					180	45*	100**
1 hr					624	370**	462
2 hr					1105	810	761**
3 hr					1402	1106	928*
IF ABP (ng/ml)					917	887	994
Serum ABP (ng/ml)	266	245	245	223	194	231	257*
Epididymal ABP (ng/ml)	10.4	7.9	8.6	8.6	8.9	10.1	11.2
Seminiferous tubular ABP (ng/ml)					1871	2452	2064
Testicular glutathione (μm/g)					2.56	2.52	2.52

[a] T, testosterone; IF, interstitial fluid; ABP, androgen-binding protein.
* $p < 0.01$.
** $p < 0.05$.

TABLE 6

EFFECTS OF *IN UTERO* AND LACTATIONAL EXPOSURE OF THE DAM TO METHOXYCHLOR AT 50 mg/kg/day ON THE REPRODUCTIVE FUNCTION OF THE FEMALE OFFSPRING

	Control	Methoxychlor
Age at Vaginal Opening	32.5 (18)[a]	26.3 (27)*
Fertility (%)	100 (10)	70 (10)
No. pups/female (days 35–253)	78.3	33.0*
No. litters/female	6.7	3.4*
Average litter size	11.8	9.9
Cyclicity (% normal at 11 months)	60	0*
Body weight at necropsy (g)	346.7	348.6
Liver weight (g)	13.2	17.2*
Paired adrenal weight (mg)	73	90*
Ovarian weight (mg)	64	69
Right kidney weight (g)	1.40	1.62**
Pituitary weight (mg)	15.8	16.2
Pituitary abnormalities, discoloration or tumor (%)	0	50*

[a] Mean (No. observed).
* $p < 0.01$ by t test or Fisher's exact test.
** $p < 0.05$.

highest dose level used here resulted in a minimal alteration of spermatogenesis; the males were fertile, but epididymal sperm storage was markedly altered. Perinatal administration of methoxychlor to the dam altered sex differentiation in the female fetus such that fertility was reduced and the onset of constant estrus in middle age was accelerated.

The acceleration of vaginal opening, followed by persistent estrus, is the clearest demonstration of the estrogenicity of methoxychlor, as cornification of the vaginal epithelium is a standard bioassay for estrogens. In fact, it was apparent within 3 days of treatment that vaginal opening in the female rat was accelerated, even at the lowest dose. The age at vaginal opening in the rat is often used in developmental studies as the sole index of puberty. However, as the results of the present study clearly show, vaginal opening is not necessarily linked with the onset of cyclicity. In the females administered 200 mg/kg/day methoxychlor, vaginal opening was accelerated but the onset of estrous cyclicity was delayed, and they were infertile. It is likely that this weak estrogen is acting directly on the vaginal epithelium of the prepubertal female. The lowest dose (25) did accelerate puberty as indicated by both measures, intermediate doses (50 and 100) had no effect, and puberty was delayed at the high dose (200). The intermediate doses of methoxychlor (50 and 100) also prolonged cycle length. The number of days of cornified smears was increased and the number of leukocytic smears (characteristic of metestrus and diestrus 1 and 2) was reduced, often to a single day.

The alterations of pubertal development and estrous cyclicity occurred at doses below those that altered fertility in the female rat. The two highest doses (100 and 200) significantly reduced fertility, while litter size was marginally reduced ($p < 0.1$) at 50 mg/kg/day. In the first block, when treated males were bred with treated females, both at 100 mg/kg/day, 12 of 15 were sperm positive, but only 3 of the 12 became pregnant. The other 9 females remained in constant estrus and did not even become pseudopregnant. This indicates that the treated males did not provide sufficient copulatory stimulation to induce pseudopregnancy. Similar effects were seen at 200 mg/kg/day, but none of those females were fertile. When treated females were paired with untreated males, all of the females either became pregnant or pseudopregnant, indicating that the cervical stimulation provided by the untreated male during mating induced at least a minimal level of ovulation, corpus luteum formation, and progesterone secretion in the constant-estrus females. These events are dependent on hypothalamic–pituitary release of an LH surge (for ovulation) and prolactin (essential to maintain the corpus luteum during pseudopregnancy). However, none of the high-dose-group females became pregnant due to failure of either sperm transport and fertiliza-

tion or implantation. This could be a direct effect of methoxychlor on the uterus, as suggested by Cummings and Gray (1987), or the failure of fertilization could be due to inhibition of sperm transport to the oviducts or acceleration of the flow of ova through the oviducts. However, the data also indicate that ovarian luteal function is abnormal. Ovarian weight was reduced, and this generally reflects a reduction in corpus luteum formation. Upon observation of the ovaries at necropsy we found that five of eight of these females had no visible corpus luteum on the surface of the ovary—an observation supported by the histology data. Similar ovarian effects develop after local application of estradiol to the ovaries. Dierschke *et al.* (1983) found that injection of estradiol results in a 50% reduction in the number of ovulations by inducing atresia of preovulatory follicles. Recent work by Cummings (in press) demonstrates that short-term administration of methoxychlor prior to implantation blocks this event, while treatment after implantation inhibits luteal function, lowering serum progesterone levels and resulting in fetal death.

The physiological alterations seen in the female at 50 mg/kg/day are not severe enough to block fertility when administered from weaning through puberty, gestation, and lactation, but the process of sex differentiation of the offspring, exposed *in utero* and during lactation, is permanently affected. These females are defeminized by perinatal exposure to this weak estrogen and produced 50% fewer offspring than did controls. In addition, reproductive aging is accelerated. At 11 months of age 60% of the controls are still cycling normally, but the treated female offspring are in constant estrus or are pseudopregnant. No statistically significant abnormalities were noted in the male offspring.

In the male, we measured preputial separation because it is the only noninvasive measure of puberty in the male rat. This androgen-dependent event was delayed by methoxychlor treatment in at 100 and 200 mg/kg/day. The serum testosterone levels at necropsy were undetectable in the males in the high-dosage group in the first block and we found a reduced response to *in vitro* hCG stimulation in the testes of treated males in the third block. These results indicate that Leydig cell function was inhibited by methoxychlor treatment in the absence of an effect of pituitary or serum LH. The males with the longest delay in PPS also displayed the most severe alterations of epididymal function and spermatogenesis. Preputial separation never occurred in the male with minimal tubular atrophy and his total cauda epididymal sperm content was the most severly affected of any of the methoxychlor-treated males, having 568,000 sperm as compared to the control mean of 78,000,000. The histology of the testis of the second-most severly affected male was not affected even though he had only 11,000,000 sperm in the cauda and PPS did not occur until he was 58 days old, an 18-day delay. Other researchers of delayed preputial separation in the rat have also observed serious functional reproductive deficits as well, indicating that a significant delay in this simple endpoint may have considerable utility in the identification of reproductive toxicants that alter endocrine function. In the male rat, pituitary prolactin levels were elevated, even at the lowest doses of methoxychlor, while other endocrine, sperm, and testicular measures were unaffected. It is well known that estrogen treatment induces hyperprolactinemia in the rat and this results in abnormal pubertal development and infertility. However, the levels of serum prolactin seen here were not significantly elevated and represent relatively modest changes as compared to the increases in prolactin associated with long-term estrogen treatment and infertility in the male rodent.

Although methoxychlor treatment did not cause testicular atrophy in the present study, other studies have reported that dietary administration of 1–3% methoxychlor resulted in severe testicular atrophy (Tullner and Edgcomb, 1962; Hodge *et al.*, 1950) and hypospermatogenesis, effects that were not found in pair-fed controls. These doses also resulted in about a 60% decrease in the final body

weight as compared to a 32% reduction seen here in the highest dosage group (200). In the current investigation, cauda epididymal weight, sperm reserves, and seminal vesicle weight were significantly reduced at 50 mg/kg/day and above, but severe testicular lesions were not detected at any dose. In addition, glutathione levels, a potential marker of tubular function, were not depressed and testis weight was only slightly affected. The fact that these effects occur in the absence of any changes in serum hormones suggests that methoxychlor directly affects the function of these reproductive tissues. Robaire *et al.* (1987) reported similar results in adult male rats following sustained administration of estradiol from polydimethylsiloxane capsules. They found that estradiol treatment reduced caudal weight, caudal sperm count, seminal vesicle weight, serum testosterone, and fertility, but had no effect on testis weight, sperm production, or testicular morphometry (Robaire *et al.*, 1979). They postulated that the infertility was due to a suppression of male sexual behavior. While the effects of methoxychlor on Leydig cell function in the current study are concurrent with slight changes in pituitary prolactin content and secretion of this hormone *in vitro* (Goldman *et al.*, 1986), LH levels in the serum and pituitary are normal.

In addition to the reproductive tissues, most organs have steroid hormone receptors and for this reason many are also "target organs" for estrogen-induced toxicity. For example, the increase in adrenal weight, seen in Blocks 1 and 3 at 100 and 200 mg/kg/day, is not due to a "stress effect," but rather is likely a consequence of the estrogenicity of methoxychlor. In the rat the adrenal is a sexually dimorphic gland that is stimulated by estrogens (Jones, 1955).

In contrast to the multiple reproductive endpoints of male and female reproductive physiology included here, the standard multigenerational reproduction test measures only growth, fertility, litter size, and growth and viability of the offspring (Palmer, 1981). We propose that an evaluation of the reproductive toxicity of methoxychlor and other estrogens [including estradiol and diethylstilbestrol (DES)] that relies solely on the endpoints included in a standard multigeneration test would detect infertility only at "toxic" dose levels, resulting in the conclusion that estrogens are not reproductive toxicants in the rat. Growth was retarded at all dose levels in males and infertility was detected only in the two highest dosage levels, which reduced male body weight at necropsy by 23 and 32% respectively. Testicular weights were less dramatically reduced than was body weight and testicular histology was only minimally affected. However, the alteration of growth in the male rats treated with methoxychlor is not nonspecific toxicity, but is likely a direct consequence of methoxychlor's estrogenicity (Heywood and Wadsworth, 1980). Pharmacological doses of estrogens retard skeletal growth in both humans and animals. In rats, estrogens reduce growth through direct effects on the central nervous system (altering food consumption) and on metabolism (Wade, 1976). These are species-typical responses, as food consumption in other rodents and in humans is less affected than in the rat. The alterations of serum and pituitary TSH seen in the current investigation have been reported for other estrogenic compounds (Cooper *et al.*, 1986), and an alteration of pituitary–thyroid function may be responsible, in part, for the inhibitory effects of methoxychlor on growth. It is also known that estradiol inhibits growth and lowers serum somatomedin A generation, induced by growth hormone (Kawai *et al.*, 1982).

Estrogens are also human and rodent teratogens and some, like DES, are transplacental carcinogens as well. It is critical, therefore, in the assessment of reproductive risk to humans from exposure to compounds like methoxychlor, that test protocols detect estrogenicity as well as infertility. Other toxicants that lack estrogenicity cause infertility through a variety of different mechanisms and the more information that we have about the reproductive effects, the more intelligently we can extrapolate such effects to

TABLE 7

STATISTICAL PRECISION OF VARIOUS ENDPOINT MEASURES IN CONTROL MALES IN THE THIRD BLOCK

Parameter	$\bar{x}$	CV[a]	%D[b]	Parameter	$\bar{x}$	CV	%D
Body weight (g)	489.1	10	8	Serum T	2.80	76	64
Liver weight (g)	24.7	15	12	Serum TSH	6.29	59	50
Right kidney weight (g)	1.75	9	7	Serum ABP	194.5	24	21
Adrenal weight (mg)	51.4	22	19	Serum LH	0.475	42	36
Seminal vesicle weight (g)	1.73	14	12	Serum FSH	8.95	15	13
Right testis weight (g)	1.87	11	9	Serum prolactin	3.73	89	73
Pituitary weight (mg)	10.73	9	8	Pituitary FSH	592.0	18	15
Right cauda epididymal weight (g)	300.7	16	13	Pituitary LH	680.5	21	18
				Pituitary TSH	1579.3	21	18
Age at preputial separation	39.5	8	6	Pituitary prolactin	1936.4	32	28
Weight at preputial separation (g)	194.6	12	10	Coulter counter caudal sperm count ($\times 10^6$)	138.5	27	23
Testicular sperm head count ($\times 10^6$)	197.0	14	12	*In vitro* T Production			
Sperm motility	52.5	33	27	hCG 0 hr	179.7	81	68
Testicular IF[c] weight	92.4	35	30	hCG 1 hr	624.2	36	31
Testicular IFT	298.3	96	80	hCG 2 hr	1105.4	34	29
Testicular IF ABP	917.4	25	21	hCG 3 hr	1402.4	35	30
Seminiferous tubule fluid weight	97.9	60	51				
Seminiferous tubule fluid T	761.5	42	35				
Seminiferous tubule fluid ABP	2599.4	16	13				
Caput epididymal T	3.89	72	61				

[a] Coefficient of variation.
[b] Percent deviation from $\bar{x}$ required for statistical significance at 0.05 level. $(LSD/\bar{x}) \times 100$.
[c] IF, interstitial fluid; T, testosterone; ABP, androgen-binding protein.

humans. In addition, from the data presented here, it is clear that significant physiological alterations are present in the male and female at doses of methoxychlor well below those that lower fertility, indicating that the lower doses are not "no-observed-effect levels."

Not only is fertility often a biologically insensitive reproductive endpoint, but there are statistical problems associated with it as well. For example, in some strains of rats many of the untreated breeding pairs do not breed, a fact that makes it very difficult to detect subtle changes in this parameter. Furthermore, it is difficult to estimate the necessary sample size to detect significance because the proportion of fertile controls is not constant from experiment to experiment. In contrast, in the present study testicular weights differed between the control males ($n = 15$) and the males treated with 200 mg/kg/day methoxychlor ($n = 12$) by 17% (Block 1), an effect that was significant at the 0.01 level. A summary of the statistical precision of the various endpoints used in the present investigation for Block 3 is shown in Table 7. One problem with using the variance of the control group to predict sample sizes for statistical precision, as suggested by some, is that treatments often affect some animals more than others and this in turn dramatically increases the pooled variance in an ANOVA. Further-

more, it is important that investigators not confuse statistical precision with biological sensitivity; for example, even though caudal sperm count is a less precise variable than testis weight or indices of daily sperm production, caudal sperm content is more sensitive to the effects of estrogens (Robaire *et al.*, 1987).

Blazak *et al.* (1985) has reported a similar analysis of potential indicators of reproductive toxicity, using fewer endpoints. In their examination of the male F-344 rat, they found low variability in testicular sperm production, but reported that testis weight and epididymal sperm number were unreliable quantitative indicators. Certainly, this generalization does not hold true for the effects of methoxychlor or estradiol on the male rat, where epididymal sperm numbers are dramatically reduced, but testicular sperm numbers are unaffected. The epididymis is more than an efferent duct and performs many important functions. Methoxychlor, like estradiol, may hasten sperm transport through the epididymis so that the time for sperm maturation is reduced (Cooper *et al.*, 1986). In addition, we found a 11% reduction in testis weight as a result of methoxychlor treatment in the absence of an effect on sperm production. Therefore, it is important to evaluate multiple testicular and epididymal endpoints in any reproductive assessment of the male rat.

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